EXHIBIT H

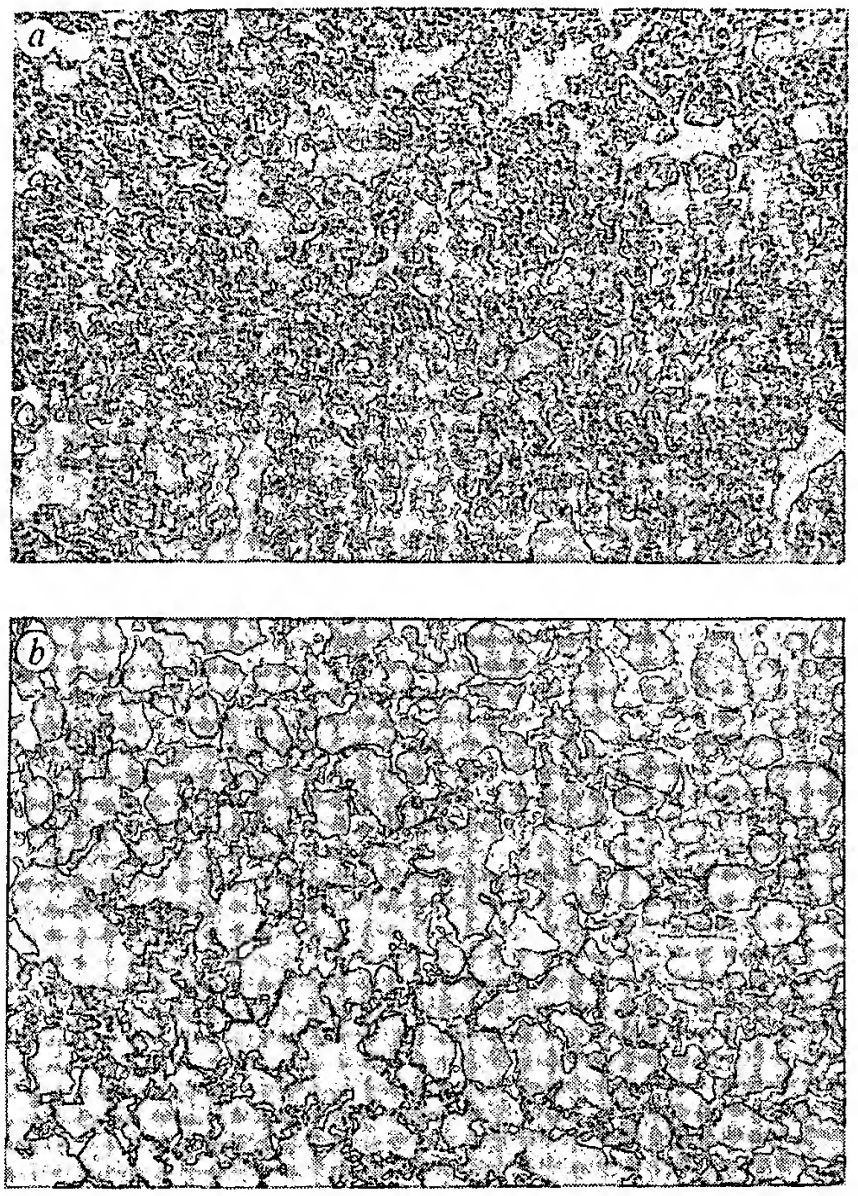

FIG. 3 Microscopic appearance (magnification, 160×) of a rabbit lung removed: *a*, immediately after death at 26 h from an animal receiving endotoxin and no IL-1ra; and *b*, 24 h after identical injection of endotoxin from an animal receiving 100 mg rhIL-1ra per kg body weight over the first 24 h. Rabbits were killed with pentobarbital. The tissue specimens were fixed in buffered (pH 7.4) 10% formalin, dehydrated and embedded in paraffin. The tissue samples were analysed with light microscopy with haematoxylin–eosin staining.

IL-1ra per kg body weight, the level of IL-1ra in plasma, as measured by a single radial immunodiffusion assay[13], varied from between 150 $\mu g\ ml^{-1}$ 5 minutes after each 7.7-mg kg^{-1} dose, and 20 $\mu g\ ml^{-1}$ 2 hours later. These results imply that the quantity of IL-1ra needed to prevent mortality would be considerably reduced if the circulation of the protein could be prolonged.

To determine the time during which IL-1 acts a pathological agent in endotoxin shock we investigated the effects on mortality of delaying the treatment with IL-1ra. When the standard treatment with IL-1ra was delayed for 1 or 2 hours after the endotoxin injection, seven of the eight animals in each group survived the 7-day period whereas three of the four animals in a group not treated with IL-1ra died within 48 hours. These results indicate that IL-1 toxicity can be reversed at least 2 hours after injecting endotoxin. Because IL-1 seems to be a late-acting agent in endotoxin shock, IL-1ra could have therapeutic as well as prophylactic properties in septic shock. This is especially important clinically, where the disease, as measured by hypotension, can be in progress before intervention can be initiated. Experiments are in progress to determine the latest time after administration of endotoxin at which the disease can be prevented.

In light of earlier results implicating tumour necrosis factor as a mediator in endotoxin shock[14–17], the demonstration that IL-1 is also an important mediator shows that the disease probably results from several cytokines acting with additive or synergistic effects. This conclusion is in accordance with IL-1 greatly potentiating the shock action of tumour necrosis factor in mice and rabbits[8,9]. Other cytokines may also contribute to the pathology of endotoxin shock. But on the basis of the current results we conclude that IL-1 plays an important part in experimental endotoxin shock in animals. It will be worthwhile investigating whether IL-1ra is of practical therapeutic benefit in human septic shock. □

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Phage antibodies: filamentous phage displaying antibody variable domains

John McCafferty*†, Andrew D. Griffiths*, Greg Winter*‡ & David J. Chiswell†

* MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
† Cambridge Antibody Technology Ltd, Daly Research Laboratories, Babraham Hall, Cambridge, CB2 4AT, UK

NEW ways of making antibodies have recently been demonstrated using gene technology. Immunoglobulin variable (*V*) genes are amplified from hybridomas or B cells using the polymerase chain reaction, and cloned into expression vectors. Soluble antibody fragments secreted from bacteria are then screened for binding activities (see ref. 1 for review). Screening of *V* genes would, however, be revolutionized if they could be expressed on the surface of bacteriophage. Phage carrying *V* genes that encode binding activities could then be selected directly with antigen. Here we show that complete antibody V domains can be displayed on the surface of fd bacteriophage, that the phage bind specifically to antigen and that rare phage (one in a million) can be isolated after affinity chromatography.

The heavy (VH) and light (VL) chain variable (V) domains of the anti-lysozyme antibody D1.3 (ref. 2) associate tightly as an Fv fragment and bind to antigen with a similar affinity to that of the parent antibody[3]. To allow expression of both domains on the same polypeptide, they were joined by a flexible linker $(Gly_4\text{-}Ser)_3$ (ref. 4), and the single-chain Fv fragment (scFv) cloned into an fd phage vector (fdCAT1) at the N-terminal region of the gene III protein (Fig. 1). The gene III protein is normally expressed at the tip of fd phage (about four copies per virion), is responsible for attachment of phage to the

‡ To whom correspondence should be addressed.

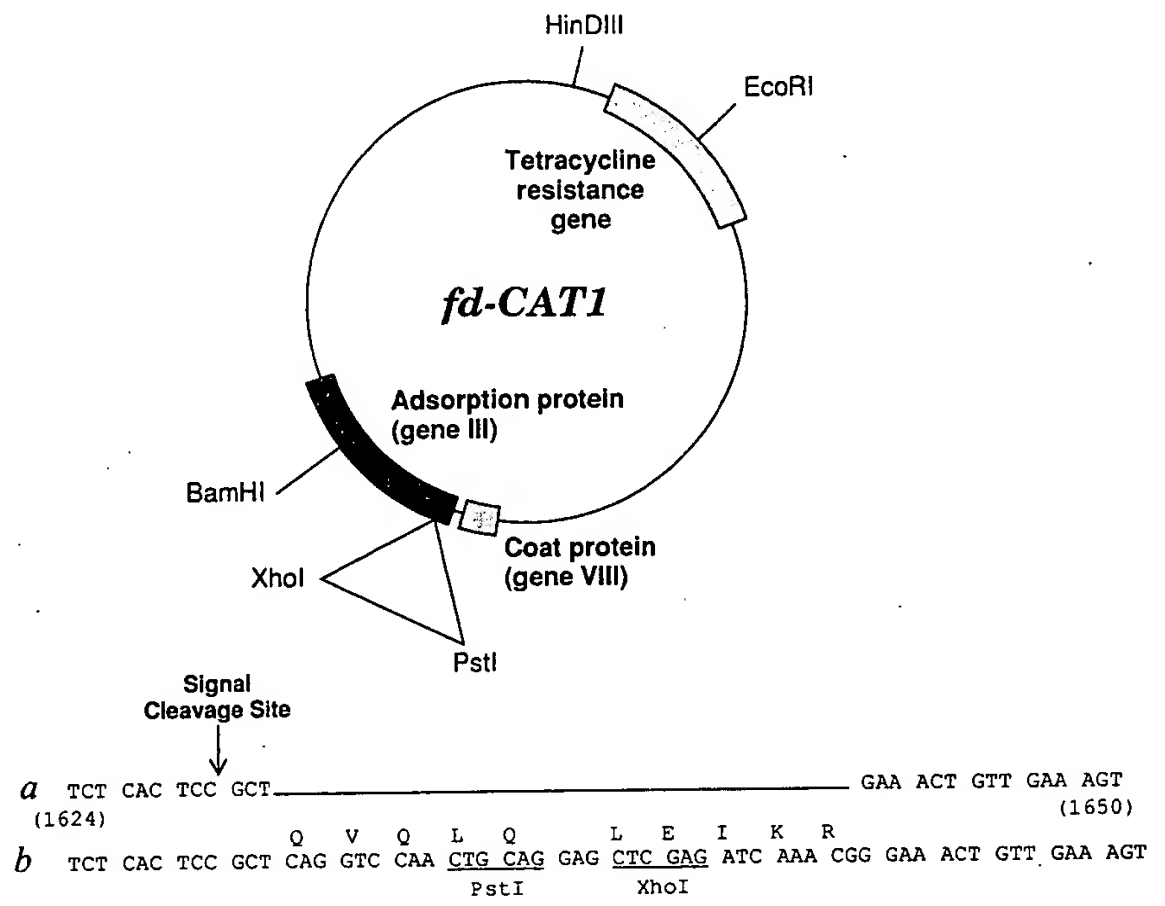

FIG. 1 Structure of vector (fdCAT1) for cloning antibody variable domains. Features of the vector used for cloning scFv (D1.3) are shown with *a*, the sequence of fd gene III around the signal peptide cleavage site in fd-tet[18], and *b*, the corresponding region of fdCAT1 with flanking immunoglobulin gene sequences.

METHODS. Vector construction: The vector fd-tet[18] from the American Type Culture Collection was used to transduce *E. coli* strain TG1=K12, (*lac-pro*), *sup*E, *thi*, *hsd*D5/F' *traD*36, *proA*+*B*+, *lacI*^{q}, *lacZ M*15) to tetracycline resistance, plating onto 2×YT plates supplemented with 15 μg ml^{-1} tetracycline[19]. First, the *Bst*EII sites of the vector were removed by digestion, fill-in and religation. To allow the cloning of the scFv (D1.3) gene between the signal sequence and N-terminus of gene III, new restriction sites (*Pst*I and *Xho*I) and flanking immunoglobulin gene sequences were introduced by site-directed mutagenesis[20]. scFvD1.3 construction. The scFv (D1.3) gene was prepared by digestion of the vector pSW2 (ref. 3) with *Bst*EII and *Sac*I, and insertion of a synthetic oligonucleotide corresponding to the 3' end of V_H, the 5' end of V_L and the peptide $(Gly_4\text{-}Ser)_3$ (ref. 4). The scFv was expressed in the bacterial periplasm, shown to bind lysozyme-coated wells by ELISA (data not shown) and then cloned into fdCAT1 at the *Pst*I and *Xho*I sites. Western blots: Concentrated phage (20 μl) (see Fig. 2 legend) was loaded on an SDS-(12.5%) polyacrylamide gel[11] and the fractionated proteins transferred electrophoretically to nitrocellulose[10]. The filter was blocked in PBS containing 5% skimmed milk powder and 0.1% Tween-20, and developed with rabbit antiserum raised against the D1.3 Fv fragment[3] and anti-rabbit antibody conjugated to horseradish peroxidase (Sigma), and detected by enhanced chemiluminescence (Amersham International). The western blots (not shown) revealed two bands (apparent relative molecular mass (M_r) 92,000 and 69,500) suggesting partial proteolysis of the fusion protein. The migration of the upper band was slower than calculated for the fusion protein (M_r 69,500), but the migration of gene III protein on SDS-polyacrylamide gels (M_r 42,100) is also slower than calculated (apparent M_r 55,000-70,000)[21].

bacterial F pilus[5], and has been used to display peptide epitopes at the surface of the phage[6-9]. The antibody-gene III fusion was detected in the recombinant phage (but not in parental fdCAT1 phage) by western blotting[10] of polyacrylamide-SDS gels[11], and probing with antisera against the D1.3 Fv fragment[3] (data not shown).

Binding of phage to lysozyme was then analysed by enzyme-linked immunosorbent assay (ELISA) (Fig. 2). The phage had the same pattern of reactivity as the D1.3 antibody[12], and bound to hen egg-white lysozyme, but not to turkey egg-white lysozyme, human lysozyme or bovine serum albumin. The specificity of the phage is particularly illustrated by the lack of binding to the turkey egg-white lysozyme, which differs from hen egg-white lysozyme by only seven amino acids[12]. The antigen-binding site is therefore displayed on the surface of the phage and retains antigen binding and specificity. We term phage that display antibody variable domains as phage antibodies.

As phage expressing small peptide epitopes can be purified from mixtures of other phage using antisera or antibodies[7-8], we attempted to purify phage antibody (D1.3) using antigen. The phage antibody was mixed with the parental fdCAT1 phage (Fig. 1 legend) and 10^{12} phage passed over a column of lysozyme-Sepharose. Colonies derived from the eluates were analysed by probing with an oligonucleotide that detects only

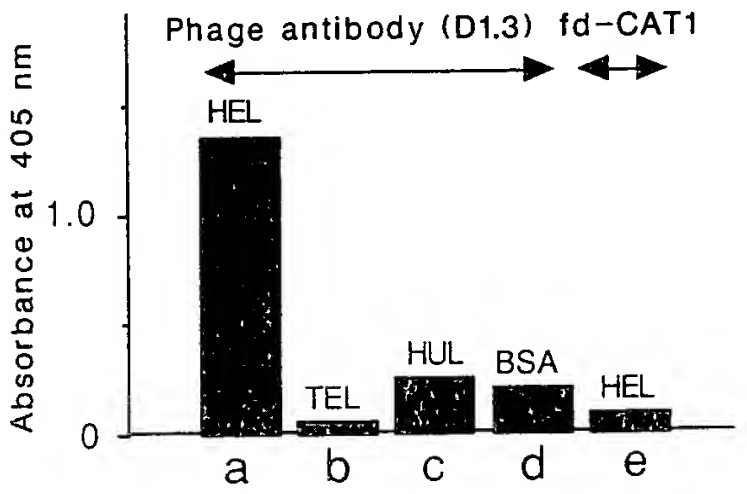

FIG. 2 Binding of phage antibody (D1.3) to lysozymes. Binding of phage as detected by ELISA to hen egg-white lysozyme (a, HEL), turkey egg-white lysozyme (b, TEL), human lysozyme (c, HUL), BSA (d). A further control was binding of fdCAT1 to HEL (e).

METHODS. Phage growth: Cultures of phage-transduced bacteria were prepared in 10-100 ml 2×YT medium with 15 μg ml^{-1} tetracycline and grown with shaking at 37 °C for 16-24 h. Phage supernatant was prepared by centrifugation of the culture (10 min at 10,000 r.p.m. in 8×50 ml rotor, Sorval RC-5B centrifuge). At this stage the phage titre was 1-10×10^{10} ml^{-1} transducing units. The phage were precipitated by adding 1/5 volume 20% polyethylene glycol, 2.5 M NaCl, leaving for 1 h at 4 °C, and centrifuging (as above). The phage pellets were resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, to 1/100th of the original volume, and residual bacteria and aggregated phage removed by centrifugation for 2 min in a bench microcentrifuge. ELISA: as described[3], except that 2×10^{10} phage transducing units were added to the antigen-coated plates (1 mg ml^{-1} antigen) in PBS containing 2% skimmed milk powder. Plates were washed between each step with three rinses of 0.5% Tween-20 in PBS followed by three rinses of PBS. Bound phage was developed by incubating with sheep anti-M13 antisera and detected with horseradish peroxidase conjugated anti-goat serum (Sigma) and ABTS (2'2'-azinobis(3-ethylbenzthiazoline) sulphonic acid). Absorbance readings were taken at 405 nm after a suitable period.

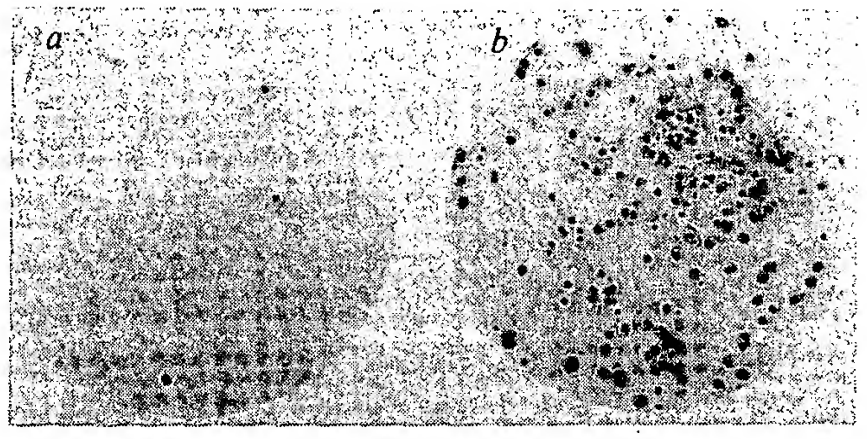

FIG. 3 Oligonucleotide probing of affinity-purified phage. Phage (10^{12}) in the ratio 1 phage antibody (D1.3) in 4×10^4 fdCAT1 phages were affinity-purified and probed with an oligonucleotide specific for phage antibody (D1.3). *a*, Filter after one round of affinity purification (900 colonies total) and, *b*, after two rounds (372 colonies total).

METHODS. Affinity chromatography of phage antibody: About 10^{12} phage particles in 1 ml 2% skimmed milk powder (MPBS) were loaded onto a 1-ml lysozyme–Sepharose affinity column which had been prewashed in MPBS. The column was washed in turn with 10 ml PBS; then 10 ml 50 mM Tris–HCl, 500 mM NaCl, pH 7.5; then 10 ml 50 mM Tris–HCl, 500 mM NaCl, pH8.5; then 5 ml 50 mM Tris–HCl, 500 mM NaCl, pH 9.5 (adjusted with triethylamine) and then eluted with 5 ml 100 mM triethylamine. The eluate was neutralized with 0.5 M sodium phosphate buffer, pH 6.8, and the phage plated for analysis. For a second round of affinity chromatography, the first column eluate was plated to about 30,000 colonies per Petri dish. After overnight growth, colonies were then scraped into 5 ml 2×YT medium, and a 20-μl aliquot diluted into 10 ml fresh medium and grown overnight. The phage was polyethylene-glycol-precipitated as above, resuspended in 1 ml MPBS and loaded onto the column, washed and eluted as above. Oligonucleotide probing. 40 pmol oligonucleotide VH1FOR (ref. 2), specific to phage antibody (D1.3) was phosphorylated and used as probe as in (ref. 19).

TABLE 1 Enrichment of phage antibody (D1.3)

Input ratio*	Output ratio		Enrichment§
	Oligo†	ELISA‡	
Phage antibody: fdCAT1	Phage antibody: total phage	Phage antibody: total phage	
Single round			
$1:4\times10^3$	43/124		1.3×10^3
$1:4\times10^4$	2/82		1.0×10^3
Two rounds			
$1:4\times10^4$	197/372		2.1×10^4
$1:4\times10^5$	90/356	3/24	1.0×10^5
$1:4\times10^6$	27/183	5/26	5.9×10^5
$1:4\times10^7$	13/278		1.8×10^6

* About 10^{12} phage with the stated ratio of phage antibody (D1.3):fdCAT1 were applied to 1 ml lysozyme–Sepharose columns, washed and eluted.

† TG1 cells were infected with the eluted specific binding phage and plated onto 2×YT plates. After incubation overnight at 30–37 °C the plates were analysed by hybridization to the oligonucleotide VH1FOR (ref. 3) which hybridizes to phage antibody (D1.3) but not to fdCAT1.

‡ Single colonies from overnight plates were grown, phage purified, and tested for lysozyme binding.

§ Enrichment was calculated from the oligonucleotide probing data.

the phage antibody (D1.3) (see Table 1 and Fig. 3). At least a thousandfold enrichment of phage antibody (D1.3) was seen with a single column pass. By growing the enriched phage and passing it down the column again, enrichments of up to a millionfold were seen.

Enrichment was also demonstrated using purely immunological criteria. For example, 10^{12} phage (in a ratio 1 phage antibody (D1.3) in 4×10^6 fdCAT1 phage) was subjected to two rounds of affinity selection, and then 26 colonies grown and the phage assayed for lysozyme binding by ELISA. Five colonies yielded phage with binding activities, and these were shown to encode the scFv (D1.3) by polymerase chain reaction (PCR) screening[13]. Thus, very rare phage antibodies can be extracted from large populations by using antigen to select and then screen the phage.

Phage antibodies are likely to find a range of applications in screening *V* genes encoding antigen-binding activities. For example, phage antibodies could be used in cloning and rescue of hybridomas[14], and in the screening of large combinatorial libraries. Thus in the 'random combinatorial' method[15], rearranged heavy and light chain *V* genes, isolated by PCR from total splenocytes of a hyperimmunized animal, are combined at random and expressed in *Escherichia coli*. As most of these *V* gene combinations are artificial, those encoding binding activities have a low frequency (1 in 3,000) (ref. 15), and probably lower affinity than original hypermutated VH–VL pairs[1]. Rounds of selection using phage antibodies may help in rescuing the higher affinity antibodies. Alternatively, phage antibodies could be used to screen small combinatorial libraries derived from antigen-selected cells[16] to rescue the original VH–VL pairs. Phage antibodies may also be of use in the construction of entirely synthetic antibodies. For example *V* gene repertoires could be made *in vitro* by combining unrearranged *V* genes with *D* and *J* segments[17]. Libraries of phage antibodies could then be selected by binding to antigen, hypermutated in the antigen-binding loops *in vitro* and subjected to further rounds of selection and mutagenesis[1,17].

The demonstration that a functional antigen-binding site can be expressed on the surface of phage has implications beyond the construction of antibodies. For example, if other protein domains could be expressed at the surface of phage, fd phage vectors could be used to clone and select genes by the binding properties of the expressed protein. Furthermore, endless variants of proteins, including epitope libraries built into the surface of the protein, could be made and readily selected for binding activities. Thus in the future other protein architectures might serve as 'nouvelle' antibodies.

Note added in proof. Insertion of the gene for a scFv directed against the hapten 2-phenyloxazolone into fdCAT1 creates a phage antibody with the predicted specificity. □

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